IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Michael A. Apicella et al.
Examiner: S. Devi
Serial No.: 09/077,572
Group Art Unit: 1641
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Title: NON-TOXIC MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA

## DECLARATION OF DRS. GIBSON AND APICELLA UNDER 37 C.F.R. § 1.132

1. We, Bradford Gibson and Michael Apicella, are two of the co-inventors of the above-identified patent application.

2. I, Bradford Gibson, am currently a full professor of Pharmaceutical Chemistry and Chemistry at the University of California, San Francisco in the School of Pharmacy, where I have been since the Fall of 1985. I have worked in the area of biomedical mass spectrometry for 20 years after receiving my doctorate in chemistry from M.I.T. and a two year postdoctoral fellowship in the chemistry department, Cambridge University, UK. I have published numerous peer-reviewed publications on Lipid A and lipooligosaccharide (LOS) structures using mass spectrometry and NMR. I have collaborated with Dr. Michael Apicella for over 10 years on the structure analysis and biology of Lipid A's and LOS from numerous pathogenic and non-pathogenic bacteria including Haemophilus, Neisseria, Salmonella, E. coli and Moraxella.

3. I, Michael Apicella, am currently a tenured Professor and Chairman of Department of Microbiology at The University of Iowa in Iowa City, Iowa. I have held that position for seven years. I began my scientific career at Johns Hopkins University in 1966 and since that time have been a faculty member of the State University of New York at Buffalo and The University of Nevada, Reno. I obtained my M.D. degree from the State University of New York in Brooklyn in 1963. I have worked in the area of Bacterial pathogenesis and genetics for the past 30 years after completing my post-doctoral fellowship at Johns Hopkins School of Medicine. I have published over 140 articles in peer reviewed scientific journals in these areas since that time. As mentioned above, I have collaborated with Dr. Bradford Gibson for over 10 years on the structure analysis and biology of Lipid A's and LOS from numerous pathogenic and non-pathogenic bacteria including Haemophilus, Neisseria, Salmonella, E. coli and Moraxella.

4. We have performed studies that determined that *H. influenzae* makes a simple truncated penta- and tetraacylated lipid A, whose structure can be derived directly from the deletion of one or two O-linked myristoyl fatty acids ($C_{14}$) from the parental lipid A structure.

5. We have performed studies that determined that in wild type strains of *Neisseria gonorrhoeae*, lipid A is hexaacylated and contains two C-12 fatty acids (lauric acid), one on each of the two glucosamines. The *htrB* mutation in *N. gonorrhoeae* strain 1291 results in the complete deletion of one of these two lauric acid moieties to form a pentaacyl lipid A structure. No fully hexaacylated lipid A species is seen, nor higher mass structures or new fatty acids. The outcome for *htrB* in *N. gonorrhoeae* is similar to the *htrB* knockout in *H. influenzae*, which produced a truncated pentaacyl and tetraacyl lipid A species.

6. In addition, some changes in the phosphorylation pattern in the LOS and lipid A moiety are observed between wild type and *htrB*- mutant in *N. gonorrhoeae* strain 1291. These changes involve an increased level of phosphoethanolamine (PEA) in both the lipid A moiety as well as the oligosaccharide.

7. We obtained a culture of the *E. coli htrB* mutant (hereinafter "the Karow strain" or "the Karow mutant") from Costa Georgopoulos, one of the co-authors of the article Karow *et al.*, J. Bact., 174:7407-7418 (1992). We then performed studies on the lipid A made by the mutant strain. In particular, we performed a mass spectrometric examination of the Karow strain. The results of this examination clearly show that the Karow strain has a set of lipid A structures different in two very important ways from the *htrb* mutant pathogens of the present invention.

8. The Karow mutant makes a fully hexaacylated lipid A structure that is distinct in mass from the lipid A made by the parental wild-type strain. Specifically, the Karow mutant appears to contain a mixture of new unsaturated fatty acids, most likely palmitoleic (C16:1) in place of the single lauric acid (C12:0) fatty acid. This substitution causes a shift up in mass of 26 and 54 Da from the major wild type lipid A (molecular weight = 1798), producing new hexaacylated lipid A molecules with molecular weights of 1824 (+26, or $C_2H_2$) and 1852 (+ 54, or $C_4H_6$).

9. Even though pentaacyl and tetraacyl substituted lipid A species are seen in addition to hexaacyl structures in Karow's *E. coli*, these structures, when present, are not simple deletions of one and two fatty acids from the wild type (as is the case for *H. influenzae htrB*), but rather contain at least one new fatty acid not present in the small amounts of corresponding pentaacyl lipid A (MW = 1588, wild type pentaacyl lipid A) seen in the wild type lipid A preparation. The molecular weights of these two lipid A molecules are 1616 and 1406, and are consistent with a loss of the palmitoleic group (-236 Da, MW 1852--> 1616, mutant pentaacyl lipid A) and then a myristic acid group (-210 Da, MW 1616--> 1406, mutant tetraacyl lipid A).

10. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements were made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

May 8, 2000
Date

Bradford Gibson

June 29, 2000
Date

Michael Apicella